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The influence of the multi-basic cleavage site of the H5 hemagglutinin on the attenuation, immunogenicity and efficacy of a live attenuated influenza A H5N1 cold-adapted vaccine virus

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ABSTRACT

A recombinant live attenuated influenza virus ΔH5N1 vaccine with a modified hemagglutinin (HA) and intact neuraminidase genes from A/Vietnam/1203/04 (H5N1) and six remaining genome segments from A/Ann Arbor/6/60 (H2N2) cold-adapted (AA *ca*) virus was previously shown to be attenuated in chickens, mice and ferrets. Evaluation of the recombinant H5N1 viruses in mice indicated that three independent factors contributed to the attenuation of the ΔH5N1 vaccine: the attenuating mutations specified by the AA *ca* loci had the greatest influence, followed by the deletion of the H5 HA multi-basic cleavage site (MBS), and the constellation effects of the AA genes acting in concert with the H5N1 glycoproteins. Restoring the MBS in the H5 HA of the vaccine virus improved its immunogenicity and efficacy, likely as a consequence of increased virus replication, indicating that removal of the MBS had a deleterious effect on the immunogenicity and efficacy of the ΔH5N1 vaccine in mice.

Introduction

The last century witnessed the emergence of three influenza pandemics that were associated with significant morbidity and mortality worldwide: the H1N1 "Spanish" influenza pandemic of 1918, the H2N2 Asian influenza pandemic of 1957, and the 1968 H3N2 pandemic that was recognized in Hong Kong. The first pandemic of this century caused by a swine origin H1N1 virus is under way. The transmission of highly pathogenic avian influenza (HPAI) H5N1 viruses from birds to humans in Hong Kong in 1997 that resulted in serious disease and fatalities (Subbarao et al., 1998; Yuen et al., 1998) alerted public health officials to the pandemic potential of HPAI H5N1 viruses because they were a novel influenza subtype to which the human population has little or no immunity. Different clades of HPAI H5N1 viruses are now endemic in various avian species (Chen et al., 2006), particularly in Southeast Asia, favoring the chances of adaptation or genetic reassortment with human influenza viruses that might lead to the acquisition of the ability of the virus to be transmissible among humans. The use of anti-viral drugs and vaccination of target populations are crucial components of intervention strategies to prevent or lessen the deleterious impact of a pandemic (Luke and Subbarao, 2006).

The development of live attenuated influenza virus vaccines (LAIV) against HPAI H5N1 viruses is one of the approaches under evaluation as part of a pandemic preparedness plan that is based on licensed technology for the production of seasonal influenza vaccines in the United States. LAIV offers potential advantages over other vaccines, notably the ability to mimic the natural route of infection

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that is associated with the induction of CD8+ T cells (Gorse et al., 1995) and mucosal immunity (Clements and Murphy, 1986). We have previously generated a recombinant LAIV ΔH5N1 vaccine where the H5 hemagglutinin (HA) and N1 neuraminidase (NA) genes were derived from A/Vietnam/1203/2004 (H5N1) (H5N1 *wt*) while the internal protein genes were from the master donor strain A/Ann Arbor/6/60 (H2N2) cold-adapted (AA *ca*) vaccine virus that confer the properties of temperature sensitivity (*ts*, poor growth at 39 °C), cold-adaptation (*ca*, efficient replication at 25 °C), and attenuation (*att*, restricted replication in the respiratory tract of ferrets) (Suguitan et al., 2006). Using site-directed mutagenesis, Jin et al. (2003) mapped the loci in the AA *ca* virus that were responsible for the *ts* phenotype: K391E, E581G, and A661T in the polymerase basic protein 1 (PB1), N265S in PB2, and D34G in nucleoprotein (NP). The H5 HA gene in the H5N1 LAIV was modified (ΔH5) to remove multiple basic amino acids at the cleavage site (MBS), a known virulence motif for poultry. The ΔH5N1/AA *ca* vaccine was shown to be safe and attenuated in chickens, mice and ferrets; although a single dose of the ΔH5N1/AA *ca* vaccine failed to elicit detectable levels of serum neutralizing or hemagglutination inhibiting antibodies against homologous and heterologous wild-type (*wt*) H5N1 viruses, immunized mice were protected from lethal challenge with homologous and heterologous *wt* H5N1 viruses (Suguitan et al., 2006). Two doses of the ΔH5N1/AA *ca* vaccine elicited high titers of neutralizing antibodies in the serum and completely protected mice and ferrets from pulmonary viral replication and systemic dissemination of homologous and heterologous *wt* H5N1 challenge viruses.

We sought to understand the molecular basis of attenuation of the ΔH5N1/AA *ca* vaccine in animal models by delineating the contributions of the modifications to the H5 HA gene, the phenotypes specified by the loci in the AA *ca* internal protein genes, and the influence of the AA genes in the context of H5N1 glycoproteins to the attenuation phenotype in chickens and mice. In addition, we evaluated the influence of the removal of the MBS on the immunogenicity and efficacy of the ΔH5N1/AA *ca* vaccine in mice.

Results

The in vitro phenotypes of the recombinant viruses

Three genetic modifications were applied to the H5N1 *wt* virus to generate the ΔH5N1/AA *ca* vaccine virus, each of which could contribute to its attenuation: (1) the MBS in the H5 HA gene was removed (Fig. 1); (2) the avian H5N1 surface glycoprotein genes were made to function in the backbone of the human AA influenza virus genes; and (3) the set of mutations specified by the AA *ca* loci that confers the *ts*, *ca* and *att* phenotypes were introduced (K391E, E581G, and A661T in PB1, N265S in PB2, and D34G in NP). To assess the relative contributions of each of these genetic components to the attenuation of the ΔH5N1/AA *ca* vaccine virus in mice, several recombinant viruses were generated (Fig. 2) and compared for their level of replication *in vitro* and *in vivo*.

H5N1 wt	CCT	CAA	AGA	GAG	AGA	AGA	AGA	AAA	AAG	AGA	↓	GGA	TTA	TTT
	Pro	Gln	Arg	Glu	Arg	Arg	Arg	Lys	Lys	Arg		Gly	Leu	Phe
ΔH5N1 wt	CCT	CAA	AGA	GAG	ACT	---	---	---	---	CGA	↓	GGA	TTA	TTT
	Pro	Gln	Arg	Glu	Thr	---	---	---	---	Arg		Gly	Leu	Phe

Fig. 1. Deletion of multiple basic amino acids in the cleavage site of the H5 HA. The nucleotide codon sequence, as well as the corresponding amino acids, around the H5 HA cleavage site of influenza A/Vietnam/1203/2004 (H5N1) *wt* virus are shown. In addition to the deletion of several basic amino acids around the cleavage site of the H5 HA of the ΔH5N1 *wt* virus (indicated by dashes), some residues were also mutagenized, preserving the amino acid sequence (underscored). An arrow indicates the site of cleavage of the HA into HA1 and HA2 subunits.

A comparison of the titers of the recombinant viruses at 25 °C and 33 °C in primary chick kidney (PCK) cells revealed that all the viruses tested were *ca* as they were able to replicate equally well at 25 °C as they did at 33 °C, with less than 100-fold difference between the respective titers at these temperatures for each virus (Table 1). These findings are in agreement with our previous observations that several *wt* human influenza viruses can replicate efficiently at 25 °C and that the *ca* phenotype is not a discriminating phenotype among all recombinant viruses (Suguitan et al., 2006). On the other hand, only the recombinant viruses possessing the AA *ca* genetic background were *ts* (Table 1), which was expected because this phenotype is specified by loci in the internal protein genes of the AA *ca* virus (Maassab and DeBorde, 1985; Jin et al., 2003; Jin et al., 2004). Viruses with an intact H5 HA replicated to higher titers (≥100-fold) at 33 °C compared to their ΔH5 HA virus counterparts (Table 1).

As expected, the recombinant viruses without the MBS in the H5 HA required trypsin to plaque efficiently in chick embryo fibroblast (CEF) cells (Bosch et al., 1981; Webster and Rott, 1987), in contrast to the efficient plaquing in the absence of trypsin displayed by recombinant viruses that had the intact H5 HA (Table 1).

The role of MBS in the attenuation of an H5N1 LAIV

To determine the contribution of the removal of the MBS to the attenuation of the ΔH5N1/AA *ca* vaccine virus, the pathogenicity of recombinant viruses that contained the H5 HA with or without the MBS on different genetic backgrounds was evaluated in mice and chickens. Simply removing the MBS from the H5 HA of the H5N1 *wt* virus rendered it non-lethal in mice and chickens (Table 2, H5N1 *wt* vs ΔH5N1 *wt*), indicating that the MBS is indeed a major virulence factor for mice and poultry, consistent with previous reports with other H5 viruses (Bosch et al., 1979; Kawaoka and Webster, 1988; Hatta et al., 2001). ΔH5N1 *wt*-infected chickens had detectable levels of influenza-specific antibodies on day 14 post-infection (p.i.), indicating that this recombinant virus replicated in the animals. Oropharyngeal swabs from all chickens infected with the ΔH5N1 *wt* virus and cloacal swabs from 5 out of 8 of these chickens tested positive for virus shedding, with significantly lower ($>10^4$–fold) mean virus titers compared to the titers shed by chickens infected with the H5N1 *wt* virus (Table 2). Restoring the MBS in the ΔH5N1/AA *ca* vaccine made the virus (H5N1/AA *ca*) lethal to mice at a high dose but remained non-lethal to chickens (Table 2) either because the AA *ca* virus conferred the *ts* phenotype that restricted the level of replication of the virus at the high body temperature of chickens or because human influenza viruses are restricted in replication in chickens for other reasons. The H5N1/AA *wt* virus was virulent in mice but only 1 out of 8 chickens inoculated with this virus intravenously (i.v.) died. However, most of the birds developed mild to severe hemorrhage of the combs and shanks from which they eventually recovered. In contrast, the ΔH5N1/AA *wt* virus was lethal to mice only at a very high dose; although 1 out of 8 chickens infected i.v. with this virus died (Table 2), hemorrhage of the combs and/or shanks were not observed among the surviving chickens. No detectable virus shedding was observed in the oropharynx and cloaca of chickens infected with either H5N1/AA *ca* or H5N1/AA *wt* virus (Table 2).

The level of replication of the recombinant viruses was assessed in different organs of mice. All the recombinant viruses that possess the unmodified H5 HA (H5N1 *wt*, H5N1/AA *wt* and H5N1/AA *ca*) replicated to significantly higher titers in both the upper and lower respiratory tract of mice compared to their respective virus counterparts that lacked the MBS (ΔH5N1 *wt*, ΔH5N1/AA *wt* and ΔH5N1/AA *ca*, respectively) (Tables 3 and 4A). These results suggest that removal of the MBS in the H5 HA is independently associated with a significant reduction in the level of viral replication in the respiratory tract of mice. However, only the H5N1 *wt* virus consistently disseminated and replicated in the brain (Table 3), indicating that in the context of the

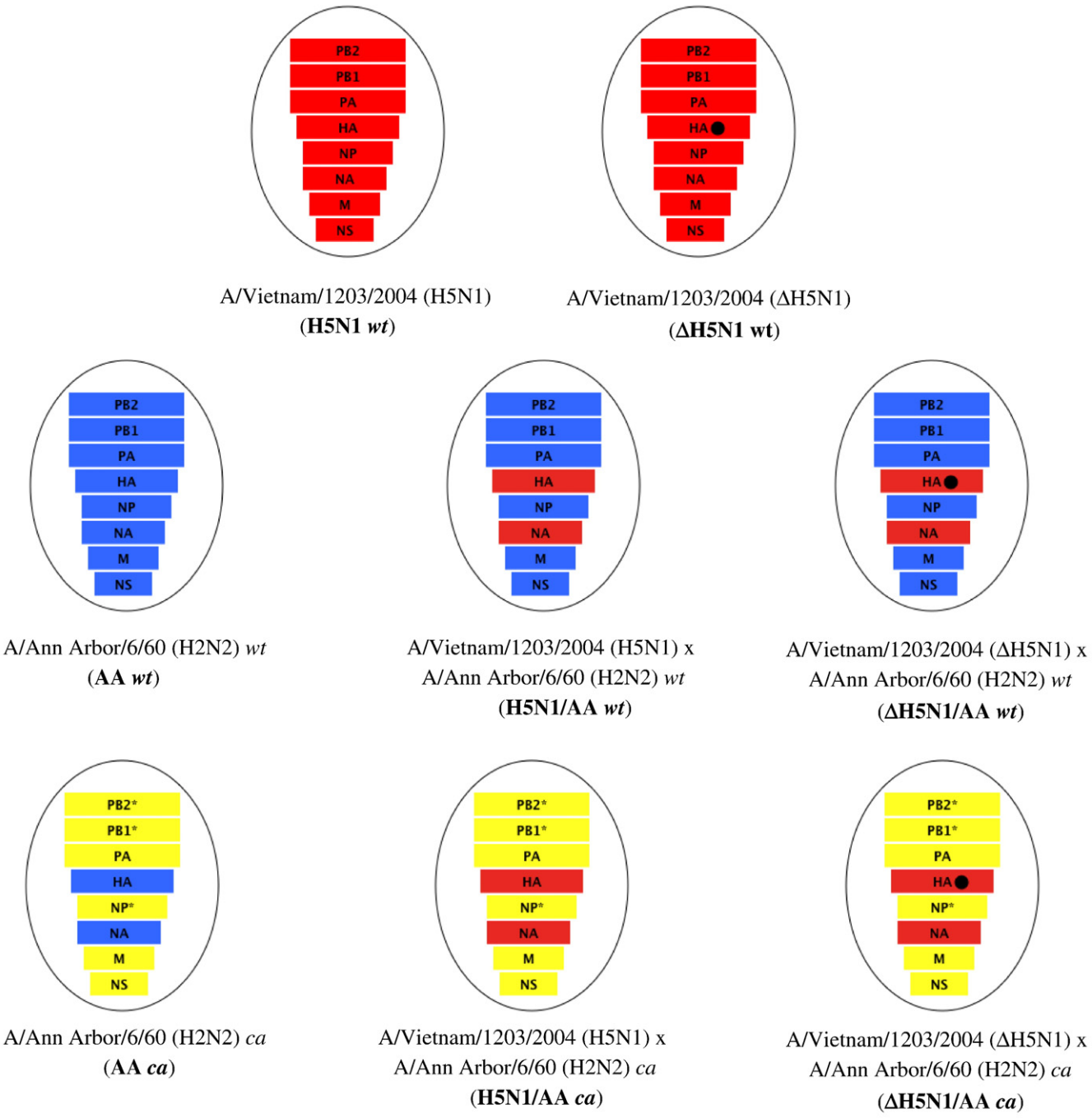

Fig. 2. Recombinant and wild-type influenza viruses used in this study. Colored bars indicate the origin of the influenza gene segment: A/Vietnam/1203/2004 (H5N1, red), A/Ann Arbor/6/60 cold-adapted virus (AA *ca*, yellow) and A/Ann Arbor/6/60 wild-type virus (AA *wt*, blue). An "●" symbol denotes that multiple basic amino acids around the H5 HA cleavage site were deleted. Asterisks indicate AA *ca* gene segments that contain loci that confer the *ts* phenotype.

H5N1 *wt* virus, the MBS in the H5 HA is a determinant of extrapulmonary dissemination.

The influence of avian H5N1 glycoproteins presented in the context of a human (AA wt) influenza virus

When the six internal protein gene segments of the H5N1 *wt* virus were replaced with those of the AA *wt* virus, the recombinant H5N1/AA *wt* virus remained highly lethal for mice but its virulence in chickens was reduced (Table 2). Given that the AA *wt* virus is a human influenza virus, the AA *wt* internal proteins might not be optimal for the efficient replication of the H5N1/AA *wt* virus in chickens. Despite the virulence of H5N1/AA *wt* virus for mice, however, the virus did not disseminate to the brain (Table 3), suggesting that extrapulmonary virus dissemination requires a highly cleavable H5 HA on an appropriate genetic background. The ΔH5N1/AA *wt* virus was slightly more virulent for mice than the nonlethal ΔH5N1 *wt* virus (Table 2). Both the H5N1 *wt* and H5N1/AA *wt* replicated to comparable levels in the lungs of mice, as did the ΔH5N1 *wt* and ΔH5N1/AA *wt* virus pair (Tables 3 and 4B). However, the titers of both H5N1 *wt* and ΔH5N1 *wt* viruses in the upper respiratory tract of mice were significantly higher than their H5N1/AA *wt* and ΔH5N1/AA *wt* virus counterparts on day 4 p.i. (Tables 3 and 4B). The ΔH5N1 *wt* virus was more infectious for chickens than the ΔH5N1/AA *wt*, as evidenced by the antibody

Table 1
Evaluation of the *ts* phenotype and the dependence on trypsin for plaque formation of recombinant influenza viruses.

Virus	Mean virus titer ± standard error[a] (log_{10} $TCID_{50}$/ml)			*ts*[b]	*ca*[c]	Requires trypsin to plaque on chick embryo fibroblast cells
	25 °C	33 °C	39 °C			
H5N1 *wt*	9.3 ± 0.3	9.2 ± 0.2	9.6 ± 0.1	−	+	−
ΔH5N1 *wt*	ND[d]	ND	ND	ND	ND	+
H5N1/AA *wt*	8.9 ± 0.4	9.0 ± 0.1	8.9 ± 0.1	−	+	−
ΔH5N1/AA *wt*	6.3 ± 0.3	6.3 ± 0.1	5.9 ± 0.3	−	+	+
H5N1/AA *ca*	9.3 ± 0.3	9.1 ± 0.1	5.5 ± 0.1	+	+	−
ΔH5N1/AA *ca*	6.7 ± 0.2	7.0 ± 0.1	5.0 ± 0.1	+	+	+
AA *wt*	7.7 ± 0.2	7.8 ± 0.4	7.7 ± 0.2	−	+	+
AA *ca*	8.0 ± 0.6	7.8 ± 0.1	5.3 ± 0.1	+	+	+

[a] Data represent the average and standard error from 3 replicates in primary chick kidney cells.
[b] The *ts* phenotype was determined by virus titration in primary chick kidney cells at 33 °C and 39 °C. A virus is considered *ts* when the difference in the mean titer of the virus at 33 °C and 39 °C is 100-fold or more.
[c] The *ca* phenotype was determined by virus titration in primary chick kidney cells at 25 °C and 33 °C. A virus is considered *ca* when the difference in the mean titer of the virus at 25 °C and 33 °C is less than 100-fold.
[d] ND—not determined.

response; however 1 of 8 chickens inoculated with the ΔH5N1/AA *wt* died (Table 2). These results suggest that the influence of the H5 HA MBS on attenuation is also dependent on gene constellation and recombinant ΔH5 viruses in which the internal protein genes were derived from the H5N1 *wt* virus replicated more efficiently than viruses in which the internal protein genes were derived from the AA *wt* virus. The H5N1 surface glycoproteins are pathogenic in mice and chickens when they are presented in an avian influenza virus background (H5N1 *wt*), and are lethal in mice when presented in a human influenza virus background (H5N1/AA *wt*).

The contribution of mutations in the internal protein genes of the AA ca virus

To evaluate the contribution of the mutations in the AA *ca* internal protein genes to the attenuation of the ΔH5N1/AA *ca* vaccine virus, the virulence in mice and chickens of recombinant viruses on the AA *ca* genetic background were compared to that of corresponding viruses on the AA *wt* virus background. The recombinant viruses with the AA *ca* internal protein genes were generally non-lethal to mice and chickens while recombinant viruses on the AA *wt* genetic background displayed varying degrees of lethality (Table 2). The AA *wt* and ΔH5N1/AA *wt* viruses were rendered non-lethal in mice by the introduction of the mutations associated with the *ts* and *att* phenotypes of the AA *ca* virus, while the lethality of the H5N1/AA *wt* virus was reduced by 10,000-fold on the AA *ca* background (Table 2). As discussed previously, viruses on the AA *ca* genetic background were restricted in replication in chickens either because the core body temperature of 40 °C restricted the replication of the *ts* viruses or because human influenza viruses replicate poorly in chickens. The restricted replication of these viruses explains the absence of detectable anti-influenza antibodies in chickens on day 14 p.i. (Table 2). Intriguingly, influenza-specific antibodies were not detected in any of the chickens inoculated intranasally (i.n.) or i.v. with the ΔH5N1/AA *wt* virus either, suggesting that this recombinant virus also grows poorly in chickens.

The level of replication of the recombinant viruses in different mouse organs was also compared. With one exception (day 4 p.i. H5N1/AA *wt* vs H5N1/AA *ca*), recombinant viruses on the AA *ca* genetic background replicated to significantly lower titers in the

Table 2
Virulence of recombinant influenza viruses in mice and chickens.

Virus	LD_{50} in mice[a] (log_{10} $TCID_{50}$)	Pathogenicity in chickens following virus administered by indicated route							
		Mortality (dead/total)		Antibody (detected/total)		Virus Isolation and Titer in			
						Oropharynx		Cloaca	
						Number of shedding/total	Mean titer (log_{10} EID_{50})	Number of shedding/total	Mean Titer (log_{10} EID_{50})
		i.v.[b]	i.n.[c]	i.v.	i.n.	i.n. inoculation			
H5N1 *wt*	0.4	8/8	8/8	NA[d]	NA	8/8	7.1	8/8	6.2
ΔH5N1 *wt*	>6.5[e]	0/8	0/8	8/8	8/8	8/8	2.6	5/8	1.5
H5N1/AA *wt*	1.5	1/8[f]	0/8[f]	7/7	6/8	0/8	≤0.9	0/8	≤0.9
ΔH5N1/AA *wt*	5.5	1/8	0/8	0/7	0/8	0/8	≤0.9	0/8	≤0.9
H5N1/AA *ca*	5.5	0/8	0/8	1/8	0/8	0/8	≤0.9	0/8	≤0.9
ΔH5N1/AA *ca*[g]	>6.5	0/8	0/8	0/8	0/8	0/8	≤0.9	0/8	≤0.9
AA *wt*	4.8	ND[h]	ND	ND	ND	ND	ND	ND	ND
AA *ca*[i]	>6.5	0/8	0/8	5/8	0/8	0/8	≤0.9	0/8	≤0.9

[a] Fifty percent mouse lethal dose (LD_{50}) expressed as log_{10} $TCID_{50}$ of virus. Groups of 5 female BALB/c mice were infected with serial ten-fold dilutions of virus and the dose of virus that resulted in 50% lethality was calculated by the Reed and Muench (1938) method.
[b] Groups of eight 4-week-old White Plymouth Rock chickens were inoculated i.v. with a dose of 0.2 ml of a 1:10 dilution of stock virus. The chickens were monitored for morbidity and mortality up to 10 days p.i.
[c] A separate group of chickens were inoculated i.n. with 10^6 $TCID_{50}$ of the reassortant viruses to evaluate their infectivity through a simulated natural route of infection.
[d] NA—not applicable; all chickens infected with H5N1 *wt* were dead by day 2 p.i.
[e] An endpoint titer was not achieved.
[f] 6/8 (i.n.) and 6/8 (i.v.) birds developed mild to severe hemorrhage of comb and/or shanks but eventually recovered.
[g] Data adapted from Suguitan et al. (2006).
[h] ND—not determined.
[i] Data adapted from Chen et al. (2003).

Table 3
Level of virus replication in nasal turbinates, lungs, and brain of mice.

Virus	Mean virus titer ($\log_{10}$ $TCID_{50}$/g) in indicated tissue[a]					
	Nasal turbinates		Lungs		Brain	
	Day 2 p.i.	Day 4 p.i.	Day 2 p.i.	Day 4 p.i.	Day 2 p.i.	Day 4 p.i.
H5N1 *wt*	4.5±0.2	6.5±0.3	8.1±0.2	8.1±0.1	1.9±0.2	5.4±0.3
ΔH5N1 *wt*	3.5±0.1	5.2±0.2	7.3±0.3	7.1±0.1	≤1.5±0	≤1.5±0
H5N1/AA *wt*	6.1±0.2	5.1±0.4	8.2±0.4	7.9±0.2	≤1.5±0	≤1.5±0
ΔH5N1/AA *wt*	3.7±0.4	3.6±0.1	6.8±0.3	6.9±0.2	≤1.5±0	1.6±0.1
H5N1/AA *ca*	4.1±0.2	5.4±0.3	5.1±0.4	5.1±0.2	≤1.5±0	≤1.5±0
ΔH5N1/AA *ca*	2.0±0.3	2.6±0.1	3.6±0.1	2.8±0.3	≤1.5±0	≤1.5±0
AA *wt*	6.5±0.2	5.8±0.1	8.0±0.2	7.5±0.2	≤1.5±0	≤1.5±0
AA *ca*	4.3±0.4	4.2±0.2	3.8±0.2	5.6±0.5	≤1.5±0	≤1.5±0

[a] Groups of 8 female BALB/c mice were infected i.n. with 10^6 $TCID_{50}$ of the indicated virus. Four mice were euthanized on day 2 p.i. and four were euthanized on day 4 p.i. Organs were harvested, homogenized and titered on MDCK cells. The limit of detection for the assay was $10^{1.5}$ $TCID_{50}$/g.

upper respiratory tract compared to recombinant viruses on the AA *wt* background (Tables 3 and 4C). However, the difference in viral replication was more pronounced in the lower respiratory tract where viruses with the AA *ca* internal protein genes generally replicated to about 100- to 1000-fold lower titer than viruses possessing the AA *wt* internal protein genes (Table 4C). These findings are consistent with the *ts* phenotype displayed by viruses on the AA *ca* genetic background, where viral replication occurs in the cooler region of the nasal passages but is restricted at the core body temperature of the lungs. Thus, the mutations in the AA *ca* internal protein genes contribute to the attenuation of the ΔH5N1/AA *ca* vaccine by restricting viral replication in the respiratory tract of mice.

In summary, the additive effects of the genetic modifications engineered into the H5N1 *wt* virus to generate the ΔH5N1/AA *ca* vaccine contributed to the attenuation displayed by the ΔH5N1/AA *ca* vaccine in mice ($10^{5.3}$ $TCID_{50}$/g reduction in virus titers in the lungs on day 4 p.i.; H5N1 *wt* vs ΔH5N1/AA *ca*). The attenuating mutations in the AA *ca* internal protein gene segments exerted the greatest influence on attenuation ($10^{4.1}$ $TCID_{50}$/g reduction in pulmonary virus titers, ΔH5N1/AA *wt* vs ΔH5N1/AA *ca*, Table 4C), followed by the removal of the MBS in the H5 HA ($10^{1.0}$ $TCID_{50}$/g reduction in pulmonary virus titers, H5N1 *wt* vs ΔH5N1 *wt*, Table 4A) and with a minor contribution from gene constellation effects ($10^{0.2}$ $TCID_{50}$/g reduction in pulmonary virus titers, ΔH5N1 *wt* vs ΔH5N1/AA *wt*, Table 4B).

Table 4
Genetic determinants of attenuation of LAIV H5N1 vaccine in mice.

Viruses compared to define role of:	Reduction of replication between indicated viruses in the respiratory tract ($\log_{10}$ $TCID_{50}$/g)			
	Nasal Turbinates		Lungs	
	Reduction[a]	*p* value[b]	Reduction	*p* value
A. H5 HA MBS in replication				
H5N1 *wt* vs ΔH5N1 *wt*	1.3	0.02	1.0	0.02
H5N1/AA *wt* vs ΔH5N1/AA *wt*	1.5	0.03	1.0	0.02
H5N1/AA *ca* vs ΔH5N1/AA *ca*	2.8	0.02	2.3	0.02
B. Mixing H5N1 and AA wt genes in replication				
H5N1 *wt* vs H5N1/AA *wt*	1.4	0.03	0.2	0.20
ΔH5N1 *wt* vs ΔH5N1/AA *wt*	1.6	0.02	0.2	0.19
C. AA ca genes in replication				
AA *wt* vs AA *ca*	1.6	0.02	1.9	0.02
H5N1/AA *wt* vs H5N1/AA *ca*	+0.3[c]	0.77	2.8	0.02
ΔH5N1/AA *wt* vs ΔH5N1/AA *ca*	1.0	0.02	4.1	0.02

[a] Reduction in virus titer on the indicated tissue on day 4 p.i.
[b] Mann–Whitney *U* test; $p<0.05$ is considered statistically significant.
[c] The virus titer of H5N1/AA *ca* is greater than H5N1/AA *wt* in the nasal turbinates.

The influence of MBS on the immunogenicity and efficacy of the ΔH5N1/AA ca vaccine

Because a significant contribution of the H5 HA MBS to the attenuation of the ΔH5N1/AA *ca* vaccine was observed, the influence of MBS on immunogenicity and efficacy was further examined. Mice that received a single dose of the vaccine i.n. were serially bled once a week for 8 weeks and serum neutralizing antibody titers were measured. Although neither the H5N1/AA *ca* nor the ΔH5N1/AA *ca* viruses elicited appreciable serum neutralizing antibody titers until day 28 post-vaccination (p.v.), the H5N1/AA *ca* virus was more immunogenic than the ΔH5N1/AA *ca* vaccine from that time point onward (Fig. 3). To evaluate the efficacy of a single dose of vaccine, immunized mice were challenged with 10^5 $TCID_{50}$ of the H5N1 *wt* virus on day 56 p.v. and viral titer was determined in the lungs on days 2 and 4 following challenge. A single dose of neither the ΔH5N1/AA *ca* nor H5N1/AA *ca* vaccine was able to completely protect mice from pulmonary viral replication of the H5N1 *wt* virus (Fig. 4A). Although mice immunized with a single dose of the ΔH5N1/AA *ca* vaccine had significantly lower pulmonary virus titers than unimmunized mice, the titers remained high on days 2 ($10^{7.4}$/g) and 4 ($10^{5.8}$/g) p.i. In contrast, mice that received a single dose of the H5N1/AA *ca* vaccine were better protected from pulmonary viral replication of the H5N1 *wt* virus than mice that received the ΔH5N1/AA *ca* vaccine, with lower virus titers on days 2 ($10^{6.1}$/g) and 4 ($10^{3.0}$/g) p.i. (Fig. 4A). The level of replication of the challenge virus in H5N1/AA *ca*-immunized mice was significantly lower than that seen in ΔH5N1/AA *ca*-immunized mice ($p<0.05$). Moreover, mice immunized with a single dose of the H5N1/AA *ca* virus had no detectable shedding of the H5N1 *wt* challenge virus in the upper respiratory tract on day 4 p.i. (data not shown). Two doses of either ΔH5N1/AA *ca* or H5N1/AA *ca* virus administered 4 weeks apart completely protected mice from pulmonary viral replication (Fig. 4B). A second dose of ΔH5N1/AA *ca* or H5N1/AA *ca* virus vaccine boosted serum neutralizing antibody titers, and comparable titers were achieved in mice immunized with either virus 4 weeks after the administration of the second dose (data not shown). These findings suggest that removal of the H5 HA MBS significantly restricted the replication of the vaccine virus in mice and

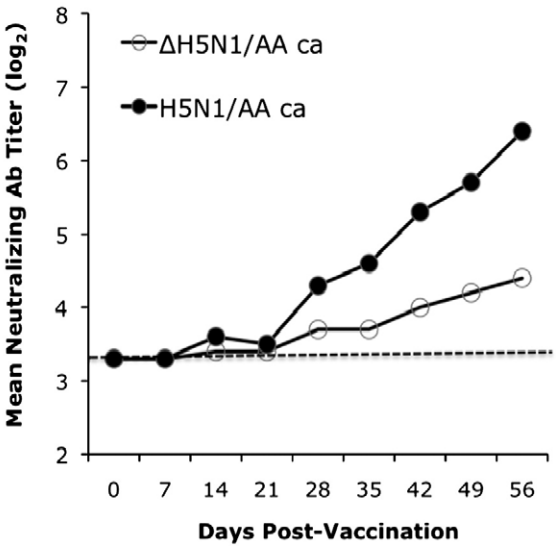

Fig. 3. Influence of the H5 HA MBS on the immunogenicity of the ΔH5N1/AA *ca* vaccine virus. Groups of 8 female BALB/c mice were immunized i.n. with 10^5 $TCID_{50}$ of either ΔH5N1/AA *ca* or H5N1/AA *ca* virus. Serum samples were collected weekly and tested in a neutralization assay against the H5N1 *wt* virus. Data represent geometric mean neutralizing antibody titers for each group. The limit of detection for the assay is indicated by the dashed line.

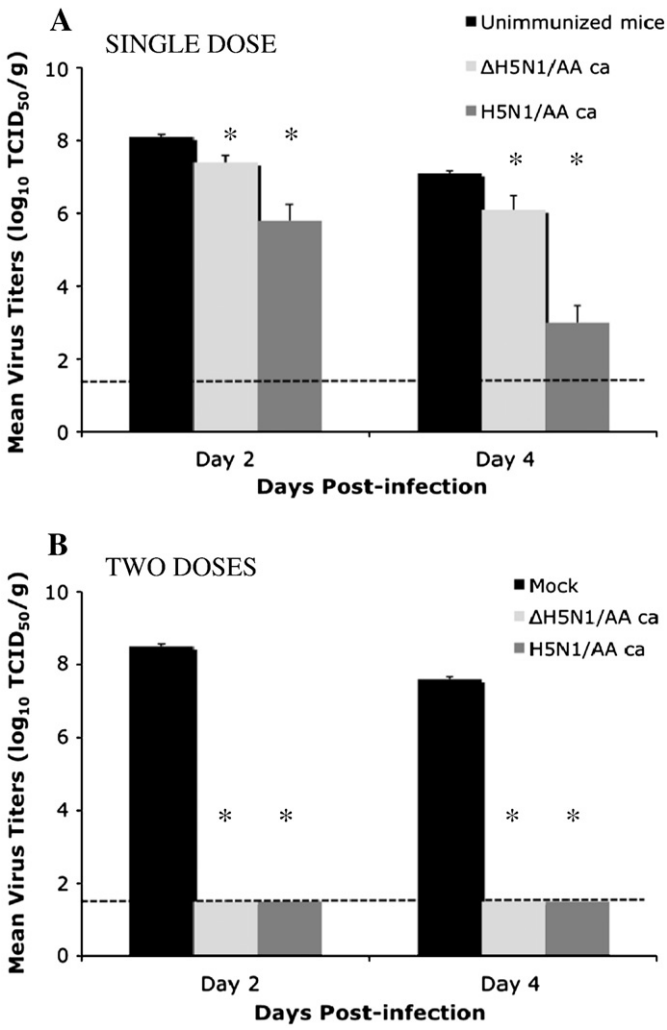

Fig. 4. Influence of the H5 HA MBS on the efficacy of ΔH5N1/AA *ca* LAIV vaccine. Groups of eight female BALB/c mice were immunized i.n. with a single dose of 10^5 $TCID_{50}$ of either ΔH5N1/AA *ca* or H5N1/AA *ca* virus (A) or two doses of the viruses 4 weeks apart (B). Mice were challenged on day 56 p.v. with 10^5 $TCID_{50}$ of H5N1 *wt* virus. Lungs were harvested on days 2 and 4 following challenge. *$p<0.05$ by Mann–Whitney *U* test, compared to the mock-immunized group.

was associated with a deleterious effect on the immunogenicity and efficacy of the ΔH5N1/AA *ca* LAIV, but this effect was overcome by administration of two doses of the vaccine virus.

Discussion

More than a decade after highly pathogenic H5N1 avian influenza virus infections were identified in humans, we cannot predict whether an H5N1 influenza pandemic will occur. HPAI H5N1 viruses are now endemic in poultry in several countries and vaccination is the most effective strategy to protect from an influenza pandemic. Therefore, the development of safe and efficacious vaccines against HPAI H5N1 viruses remains a public health priority.

Ideally, an LAIV should replicate in the host to elicit cellular and humoral immune responses without causing disease and a balance between attenuation and immunogenicity must be achieved. We developed an H5N1 LAIV vaccine using the technology employed to manufacture the licensed seasonal LAIV vaccine that relies on the *ts* and *att* phenotypes specified by the five loci in the three internal protein gene segments of the AA *ca* master donor virus to ensure that the vaccine virus replicates in the cooler upper respiratory tract and is restricted in replication at the warmer temperature of the lungs. The internal protein gene segments of the AA *ca* virus imparted the *ts* and *att* phenotypes to the ΔH5N1/AA *ca* vaccine virus mainly by restricting viral replication in the lower respiratory tract. The ΔH5N1/AA *ca* vaccine virus was not robustly immunogenic in mice and ferrets, eliciting barely detectable neutralizing antibody titers 4 weeks after the i.n. administration of a single dose of vaccine (Suguitan et al., 2006).

Although the pathogenicity of avian influenza viruses is polygenic (Rott et al., 1979; Scholtissek et al., 1979), virulence in poultry is mainly influenced by the cleavability of the HA protein (Bosch et al., 1979). The HA is synthesized as a precursor protein and must undergo post-translational cleavage into two subunits for the virus to be infectious (Lazarowitz and Choppin, 1975). HPAI H5 and H7 viruses can possess an MBS in their HA, a structural feature that renders the molecule cleavable by ubiquitous proteases such as furins (Stieneke-Gröber et al., 1992), expanding the virus' tissue range and enabling systemic spread. Results from our study confirm that the H5 HA MBS is a major virulence factor; removal of the MBS rendered the highly virulent H5N1 *wt* virus nonlethal in mice and chickens and altered the virus' ability to replicate in extrapulmonary sites in mice. Like other H5N1 vaccines that are currently in development, the MBS of the H5 HA had to be removed in the LAIV vaccine virus primarily to reduce the potential threat to poultry and presumably to make the vaccine safer for human use. In the case of the ΔH5N1/AA *ca* vaccine, the *ts* phenotype and genes derived from a human influenza virus restrict its replication in chickens. One consequence of the removal of the H5 HA MBS in the ΔH5N1/AA *ca* vaccine is the attenuation of the vaccine virus, decreasing the level of replication in the upper and lower respiratory tract by about 100-fold compared to the H5N1/AA *ca* virus. This low level of replication led to a reduction in viral load associated with diminished immunogenicity and efficacy following a single dose of the ΔH5N1/AA *ca* vaccine compared to the H5N1/AA *ca* virus in mice. The higher neutralizing antibody titers elicited by a single dose of the H5N1/AA *ca* virus correlated with improved efficacy in protecting mice from pulmonary replication of the *wt* challenge virus. A live attenuated avian paramyxovirus-vectored vaccine for chickens expressing influenza H7 HA revealed a similar dependency on the presence of an MBS and cleavability of the F protein for efficient replication and optimal protective efficacy (Swayne et al., 2003; Park et al., 2006). Although two doses of either vaccine virus completely protected mice from pulmonary replication of the *wt* challenge virus, a more immunogenic H5N1 vaccine would be preferable during a pandemic when it may be difficult to administer 2 doses of vaccine and when dose-sparing will likely be needed. The influence of genetic modifications introduced into the H5N1 vaccine on cellular immune responses is not known; however, passive transfer of sera from ΔH5N1 *ca*-immunized mice with undetectable neutralizing antibodies protected naïve mice from lethal challenge with the H5N1 *wt* virus, indicating that antibodies and/or serum factors are sufficient to mediate protection against lethality in immunized mice and that humoral immunity plays a major role in this protective immune response.

Little is known about the effect of removing the H5 HA MBS on the stability of the H5 HA protein, on the oligomerization of the H5 HA and its influence on vaccine immunogenicity. Wei et al. (2008) recently reported that different recombinant H5 HA proteins vary in their ability to elicit neutralizing antibodies depending on their multimeric nature; high-molecular weight oligomers stimulated the strongest antibody response, followed by the trimeric form of the H5 HA molecule, while H5 HA monomers were poorly immunogenic. The H5N1/AA *ca* and ΔH5N1/AA *ca* vaccines likely express subtly different H5 HA molecules on their viral surface and it is possible that these nuances could influence the nature of the antibodies that they elicit.

The results presented in this study also revealed some interesting findings regarding the influence of gene constellation effects, which may include host-range restriction, on the phenotypic characteristics of recombinant human-avian influenza viruses. Substitution of the AA

wt genetic background for the H5N1 *wt* virus internal protein genes rendered the H5N1/AA *wt* virus highly lethal in mice but not in chickens, despite the presence of the MBS in the H5 HA. The lethality of the H5N1/AA *wt* virus in mice was not associated with dissemination of the virus to the brain. These findings suggest that the mammalian origin of the AA *wt* genetic background somehow attenuates the H5N1/AA *wt* virus for chickens and that a highly cleavable H5 HA requires an appropriate genetic background to disseminate to extrapulmonary sites. A study conducted by Stech and colleagues (2009) determined that even when a non-H5/H7 avirulent avian influenza virus strain acquires a polybasic cleavage site in its HA, it is not enough to render the virus highly pathogenic, indicating that determinants specified by other viral proteins or even in other regions of the HA molecule itself can have a substantial impact on its virulence. Salomon and colleagues (2006) earlier noted that avian influenza viruses with a cleavable HA requires adaptive changes in the polymerase genes to overcome the species barrier and acquire virulence in mammals. This point is exemplified by the influenza A/Hong Kong/486/97 (H5N1) virus which, although possessing the MBS in its H5 HA, remained less pathogenic in mice but becomes highly pathogenic when its PB2 gene is substituted with a PB2 gene from a virulent H5N1 strain that bears an E627K mutation (Hatta et al., 2001; Chen et al., 2007), a mutation that has been previously implicated in the host-range restriction of influenza A viruses (Subbarao et al., 1993). Although a complex interplay between gene constellation effects, glycosylation patterns in the HA and NA, and individual gene mutations ultimately determine an influenza virus' virulence (Hulse et al., 2004; Chen et al., 2007), it is also plausible that an optimal set of polymerase proteins enables the virus to replicate efficiently in its host and spread more rapidly to other organs as well. In studies of attenuation of various influenza A H3N2 and A/Puerto Rico/8/34 (H1N1) (PR8) virus recombinants, Florent (1980) determined that recombinant H3N2 viruses were virulent when all three polymerase genes (PA, PB1 and PB2) were derived from PR8, indicating that the resulting constellation endows optimal biological activity that results in enhanced viral replication and pathogenicity.

In summary, the removal of the MBS in the H5 HA and the mutations specified by the internal protein gene segments of AA *ca* make significant contributions to the attenuation of the ΔH5N1/AA *ca* vaccine in mice by restricting viral replication in the respiratory tract. However, removal of the MBS in the H5 HA also had the unintended consequence of decreasing the immunogenicity and efficacy of the ΔH5N1/AA *ca* vaccine virus, likely resulting from restricted replication. Alternative approaches to improve the immunogenicity of the H5N1 LAIV vaccine could be considered, possibly including the use of immunostimulatory components to enhance host-protective effector responses. It would also be interesting to explore the feasibility of transferring the *ts* genetic signature of the AA *ca* virus strain to the H5N1 *wt* genetic background, as has been successfully done with PR8 (Jin et al., 2004) and an H9N2 virus (Song et al., 2007). An H5N1 LAIV will only be used upon the recommendation and guidance of public health authorities in the event of a declared H5N1 pandemic, with confirmed efficient human-to-human transmission, or when a pandemic is already underway in the United States. In such a scenario, the use of an immunogenic and efficacious LAIV vaccine with an intact MBS in its H5 HA may be worth considering if the benefits far outweigh the risks posed by potential genetic reassortment with a *wt* H5N1 pandemic virus.

Materials and methods

Generation of recombinant viruses

The generation of reverse genetics-derived ΔH5N1/AA *ca* vaccine, including the removal of the MBS in the H5 HA, has been described previously (Suguitan et al., 2006). The plasmids encoding the genes of the desired recombinant viruses were transfected into a co-culture of 293T and Madin Darby canine kidney (MDCK) cells by lipofection (TransIT, Mirus Bio Corp., Madison, WI). Seed virus stocks were cloned twice and amplified in specific pathogen free eggs. All eight gene segments of the recombinant viruses were amplified by RT-PCR and the sequences were confirmed to be identical to the plasmids used in transfection.

Phenotypic analyses of the reassortant viruses

The *ca* and *ts* phenotypes of the parent and reassortant viruses were evaluated by comparing their growth and replication in PCK cells (Charles River Laboratories, USA) at 25 °C and 33 °C (for *ca* phenotype) and 33 °C and 39 °C (for *ts* phenotype). The cells that were incubated at 25 °C were examined for cytopathic effect (CPE) on day 10 p.i. while cells that were incubated at 33 °C and 39 °C were examined for CPE on day 6 p.i. as previously described (Suguitan et al., 2006). By convention, a virus was considered *ca* if the virus titer at 25 °C was within 100-fold of the titer at 33 °C and viruses that displayed 100-fold or greater reduction in titers at 39 °C compared with that exhibited at the permissive temperature of 33 °C were considered *ts* (Suguitan et al., 2006).

Trypsin-dependence assay

CEF cells were seeded to a density of 5×10^6 cells/25 cm^2 of a 6-well tissue culture plate (Multiwell Primaria, Becton Dickinson, Franklin Lakes, NJ) and incubated overnight to achieve confluency. CEF cells were then inoculated with 0.1 ml of serially-diluted virus in L-15 medium (Invitrogen, Grand Island, NY). The virus was adsorbed to cells for 1 h at room temperature. The inocula were removed and the cells were overlaid with 1.8% agar (Difco, BD Diagnostic Systems, Palo Alto, CA) supplemented with 2× Medium 199 (Sigma, St. Louis, MO), 2× antibiotics/antimycotic (Invitrogen, Grand Island, NY), with or without 0.5 μg/ml of TPCK trypsin (Sigma, St. Louis, MO). Recombinant viruses on the AA *ca* genetic background were incubated at 33 °C for 5 days while viruses on the A/Vietnam/1203/04 or AA *wt* genetic backgrounds were incubated at 37 °C for 3 days. The overlay was carefully removed and the wells were stained with crystal violet to visualize plaques.

Pathogenicity and infectivity study in chickens

The pathogenicity of the recombinant viruses was assessed through a pathotyping test where the viruses were administered i.v. to groups of eight 4-week-old White Plymouth Rock chickens at a standard dose of 0.2 ml of a 1:10 dilution of stock virus. The chickens were monitored for morbidity and mortality up to 10 days p.i. (USAHA, 1994). A separate group of chickens were inoculated i.n. with 10^6 50% tissue culture infectious doses ($TCID_{50}$) of the recombinant viruses to evaluate their infectivity through a more natural route of infection. Oropharyngeal and cloacal swabs were collected for virus isolation on day 3 p.i. or on the day of death for chickens that died before day 3 p.i. All surviving chickens were euthanized and bled on day 14 p.i. and their sera were tested for evidence of seroconversion by an agar gel precipitin assay, the prescribed initial screening test for the detection of influenza A-specific antibodies in chickens and turkeys that is recommended by the USDA-National Veterinary Services Laboratories, National Poultry Improvement Plan and World Organization for Animal Health (OIE) (Swayne et al., 2008). The assay detects antibodies 10–14 days following intranasal challenge with low pathogenicity avian influenza viruses in chickens (Beck et al., 2003). However, the limit of detection of antibodies by this assay is not known.

Viral pathogenicity in mice

To determine the 50% lethal dose (LD_{50}) of the different recombinant viruses, groups of 6- to 8-week-old female BALB/c mice were lightly anesthetized and infected i.n. with serial 10-fold dilutions of the viruses in 50 μl. Mice were monitored daily until day 14 p.i. for mortality and weight loss. Mice that lost >25% of their initial body weight were euthanized in accordance with our animal study protocol. The ability of the viruses to replicate in different organs was also examined. Groups of 8 female BALB/c mice were infected with 10^6 $TCID_{50}$ of the recombinant viruses and 4 mice from each group were euthanized on days 2 and 4 p.i. Virus titers in lungs, nasal turbinates, and brains were quantified as previously described (Suguitan et al., 2006). Samples from mice that were inoculated with recombinant viruses on the AA *ca* genetic background were incubated at 33 °C for 7 days while samples from mice that received viruses on the A/Vietnam/1203/04 or AA *wt* genetic backgrounds were incubated at 37 °C for 4 days and the cells were examined for CPE. Viral titers were expressed as $\log_{10}$ $TCID_{50}$/g of tissue and were calculated by the Reed and Muench method (1938). Log-transformed viral titers were compared using the Mann–Whitney *U* test. A p value<0.05 was considered significant.

The influence of MBS on the immunogenicity and efficacy of an H5N1 LAIV

Groups of lightly anesthetized female BALB/c mice were immunized by i.n. instillation with 10^5 $TCID_{50}$ of H5N1/AA *ca* and ΔH5N1/AA *ca* in 0.05 mL of L15. Blood samples were collected weekly from day 7 to day 56 p.v. The immunized and unimmunized age-matched control mice were challenged on day 56 p.v by intranasal inoculation with 10^5 $TCID_{50}$ of the H5N1 *wt* virus in 0.05 mL of L15 given under light anesthesia. Four mice from each group were euthanized on days 2 and 4 p.i. and lungs and nasal turbinates were harvested, homogenized and titered on MDCK cells as described (Suguitan et al., 2006). In a separate experiment, groups of female BALB/c mice immunized with two doses of L15 medium (mock), 10^5 $TCID_{50}$ of H5N1/AA *ca* or 10^5 $TCID_{50}$ of ΔH5N1/AA *ca* i.n. given 4 weeks apart were challenged with 10^5 $TCID_{50}$ of H5N1 *wt* virus on day 56 p.v. and 4 mice from each group were euthanized on days 2 and 4 p.i. and lungs and nasal turbinates were harvested and titered on MDCK cells. The MDCK cells were incubated at 37 °C for 4 days and the cells were examined for CPE.

Serologic analyses

A microneutralization assay on MDCK cells was performed as described previously (Suguitan et al., 2006). Briefly, serial two-fold dilutions of mouse sera were mixed with 100 $TCID_{50}$ of the H5N1 *wt* virus, incubated for 1 h at room temperature and then transferred in quadruplicate to MDCK cell monolayers. Neutralizing antibody titer was defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of 100 $TCID_{50}$ of the *wt* virus on MDCK cells. Infectivity was identified by the presence of CPE after 4 days of incubation at 37 °C.

All experiments involving infectious *wt* H5N1 avian influenza viruses were performed in BSL-3 containment laboratories approved by the USDA and Centers for Disease Control and Prevention. Animal experiments were approved by the National Institutes of Health Animal Care and Use Committee.

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